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NEW CYTOTOXIC LUPANE LACTONES FROM *KOKOONA OCHRACEA*

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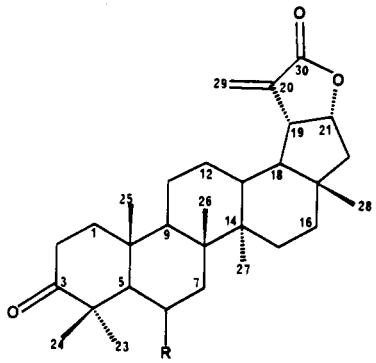
ABSTRACT.—Three new compounds, ochraceolides A [**1**], B [**2**], and C [**3**], were isolated from nonpolar extracts derived from *Kokoona ochracea* stem bark. Based on spectroscopic data, their structures were determined to be the closely related lupane lactones: 3-oxolup-20(29)-en-30,21α-olide [**1**], 20,29-epoxy-3-oxolupan-30,21α-olide [**2**], and 3,6-dioxolup-20(29)-en-30,21α-olide [**3**]. Compounds **1** and **3** exhibited significant cytotoxic activity with cultured P-388 cells (ED_{50} values of 0.26 and 0.53 μg/ml, respectively) but were at least tenfold less active with a variety of human tumor cell lines. Compound 2 was weakly active with cultured P-388 and KB-3 cells (ED_{50} values of 7.8 and 5.2 μg/ml, respectively), but no significant activity was observed with other human cancer cell types (ED_{50} values of more than 20 μg/ml).

Kokoona ochracea (Elm.) Merrill (Celastraceae) is one of eight species in the genus. It is distributed in the Malay Peninsula, Borneo, and Palawan Island of the Philippines (1). There is no local medicinal use of this plant among the Palawan inhabitants, nor could we find any documented medicinal use elsewhere. However, in Palawan at least, the tree finds application as a source of timber and fire wood. The bark contains an inflammable oil (1), and an asphalt-like residue remains after combustion. The leaves also burn readily, producing a crackling noise like that of a firecracker. Hence, the common name "Repetik" is used in Palawan.

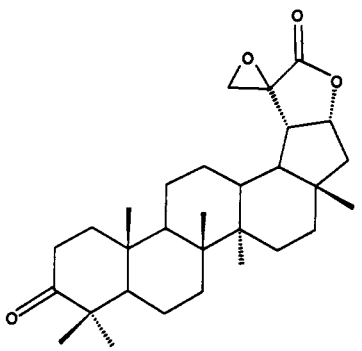
Neither the biological potential nor the phytochemical constituents of *K. ochracea* have been reported previously, except for the isolation of a cytotoxic triterpene quinone-methide, pristimerin (2). This compound had been previously isolated from two closely related species, *Kokoona zeylanica* Thwaites and *Kokoona reflexa* Thwaites, and from some other genera of Celastraceae (3,4). *K. zeylanica* and *K. reflexa* appear to be the only species in the genus that have been studied phytochemically. Oleananes and a number of friedelane triterpenes, including triterpene quinonemethides, phenolic triterpenes, and other friedo-oleananes, have been isolated from these plants (3–7). Dulcitol and 4′-*O*-methylepigallocatechin have also been isolated from the root bark of *K. zeylanica* (8).

As part of our continuing effort to identify potential antitumor agents from plant extracts, we have examined the stem bark of *K. ochracea* collected from Palawan Island. The plant material was extracted with petroleum ether, followed by MeOH and partitioning of the MeOH extract between H_2O and $CHCl_3$. The petroleum ether extract and the $CHCl_3$ phase were cytotoxic (ED_{50} values of less than 20 μg/ml), whereas the aqueous layer and the interphase were not active. The active extracts were subsequently combined and subjected to further fractionation, using UISO-BCA-1 (human breast cancer) cells to guide the isolation process. Repeated cc on Si gel and purification by crystallization led to the isolation of ochraceolides A [**1**], B [**2**], and C [**3**].

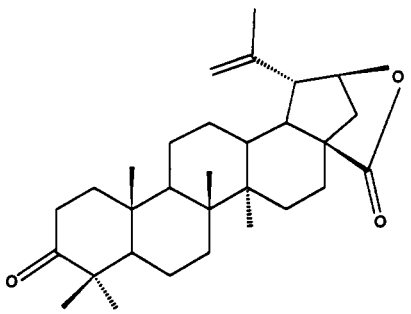
Ochraceolide A [**1**], obtained in 0.3% yield, was purified as colorless crystals ($CHCl_3$/MeOH), mp 223–225°; $[\alpha]^{25}D$ +31°. The molecular formula of **1** was assigned as $C_{30}H_{44}O_3$, based on hreims (*m/z* 452.329444; calcd 452.329045). The presence of a γ-lactone conjugated with an exomethylene was indicated by an ir absorption band at ν max 1758 cm^{-1} and a uv λ max 211 nm, as well as ^{1}H-nmr signals at δ 6.34 and 5.71 (2H, H_a-29 and H_b-29) and ^{13}C-nmr signals (Table 1) at δ 124.73 (C-29) and 139.09 (C-20), for the carbons bearing the exomethylene double bond. The lactonic carbonyl



1 R=H_2
3 R= =O



2



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signal was observed at δ 171.18 (C-30), while the carbinolic lactone carbon appeared at δ 82.17 (C-21). A proton attached to C-21 appeared at δ 4.95 as a quartet, which was subsequently shown to be a more complex coupling system by a ^1H-^1H homonuclear spin-decoupling experiment. In addition to these features, the molecule contains a ketone group at C-3, indicated by a carbon resonance peak at δ 217.78, and an ir band at 1700 cm^{-1}. Eims showed an intense fragment ion at *m/z* 205, resulting from the

TABLE 1. ^{13}C-nmr Assignments (δ, ppm) of Compounds **1**, **2**, and **3**.

Carbon	Compound			Carbon	Compound		
	1	**2**	**3**		**1**	**2**	**3**
C-1	39.51	39.29	40.77	C-16	34.52	34.02	34.24
C-2	33.99	33.84	33.65	C-17	43.84	43.27	43.66
C-3	217.78	217.80	214.19	C-18	52.17	48.74	52.02
C-4	47.22	46.99	46.73	C-19	45.04	39.66	44.92
C-5	54.71	54.36	64.90	C-20	139.09	56.79	138.80
C-6	19.57	19.39	211.04	C-21	82.17	82.03	82.02
C-7	33.40	33.16	51.73	C-22	47.75	48.03	47.47
C-8	40.79	40.60	47.36	C-23	26.68	26.54	24.03
C-9	49.23	49.16	49.64	C-24	21.00	20.80	21.61
C-10	36.80	36.58	43.34	C-25	15.93	15.82	16.30
C-11	21.17	21.16	21.08	C-26	15.70	15.55	15.89
C-12	26.49	26.31	26.42	C-27	14.09	14.12	14.40
C-13	37.73	36.58	37.24	C-28	19.34	18.93	19.22
C-14	42.99	42.76	43.26	C-29	124.73	51.94	124.77
C-15	27.67	26.40	26.96	C-30	171.18	174.06	170.97

cleavage of ring C and representing a fragment composed of rings A and B. This observation is in agreement with the fragmentation patterns of other 3-oxo triterpenes (9). Indeed, the mass spectrum of **1** was quite similar to that of thurberogenone [**4**] (9), a derivative of thurberogenin, previously isolated from the organ pipe cactus (*Stenocereus thurberi* Buxb.) (10–12). However, other spectral evidence clearly showed that **1** was not identical to thurberogenone [**4**].

The nmr spectra further indicated that **1** was a triterpene of the lupane group. It is composed of six tertiary methyls, six methines, ten methylenes, six quaternary carbons, and two carbonyl carbons. Examination of these data and comparison with those of related compounds (13–15) suggested that **1** belongs to the lupene group. Interestingly, the C-20 (δ 139.09) of **1** has undergone an upfield shift of about 11 ppm, while C-29 (δ 124.73) experienced a downfield shift of about 15 ppm, when compared with many other lupenes. This can be explained by the lactone formation at C-30 and C-21. Results from selective INEPT (16) experiments (Figure 1) were able to confirm the location of the α,β-unsaturated γ-lactone, permitting a differentiation between **1** and thurberogenone [**4**]. Thus, selective irradiation of H_a-29 (δ 6.34) and H_b-29 (δ 5.71) enhanced the carbon signals at δ 171.18 (C-30), 45.04 (C-19), and 139.09 (C-20; $^2J_{CH}$). These data confirmed that the lactonic carbonyl is at C-30, not at C-28. Magnetization transfer from H-21 (δ 4.95) led to the enhancement of signals at δ 171.18 (C-30) and 52.17 (C-18). Irradiation of H-19 (δ 3.14) enhanced the signals at δ 171.18 (C-30), 124.73 (C-29), 37.73 (C-13), 139.09 (C-20; $^2J_{CH}$), 82.17 (C-21; $^2J_{CH}$) and 52.17 (C-18; $^2J_{CH}$), the last three carbons being two bonds away from the irradiated proton. Additionally, the C-28 signal was observed at δ 19.34. These results established the location of the lactone function to be at C-30 and C-21.

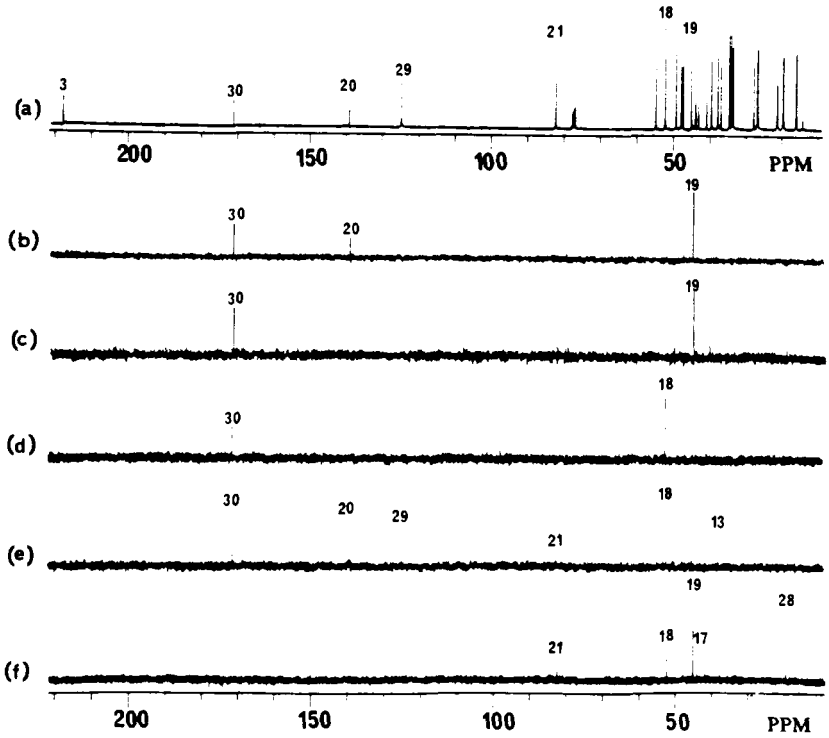

FIGURE 1. Nmr spectra of ochraceolide A [**1**]. (a) ^{13}C-nmr broad-band decoupled spectrum; (b–f) selective INEPT irradiation of (b) H_a-29, (c) H_b-29, (d) H-21, (e) H-19, and (f) H-22β.

Proton-proton connectivities were established by ^{1}H-^{1}H DQCOSY and ^{1}H-^{1}H homonuclear spin-decoupling experiments. In DQCOSY, H-21 (δ 4.95) was correlated to H-19 (δ 3.14, J = 8 Hz), H-22α (δ 1.47, J = 7.1 Hz), and H-22β (δ 2.19, J = 7.3 Hz). In addition, H-19 was coupled to H-18 (δ 1.49, J = 10.6 Hz) and showed allylic couplings with H_a-29 (δ 6.34, J = 2 Hz) and H_b-29 (δ 5.71, J = 1.7 Hz). Such assignments were confirmed by homonuclear spin-decoupling experiments, through specific irradiations at δ 3.14 (H-19), 4.95 (H-21), and 2.19 (H-22β). In order to determine the orientation of the lactone ring, a ^{1}H-^{1}H NOESY experiment was conducted. The results showed that the β-oriented Me-28 had correlations with both H-19 and H-21, indicating that H-19 and H-21 are cis- and β-oriented. On the basis of these data, the structure was established to be 3-oxolup-20(29)-en-30,21α-olide [**1**].

Ochraceolide B [**2**] was isolated as colorless crystals from Et_2O, mp 236–238°. The spectral properties of **2** were quite similar to those of **1**. However, the γ-lactone in **2** was not conjugated with an exomethylene as in the case of compound **1**. This change was noticed in its ir spectrum, in which the lactone carbonyl band shifted to a higher frequency at 1778 cm^{-1} (saturated γ-lactone). Moreover, no olefinic protons were observed in the ^{1}H-nmr spectrum of **2**; instead, an AB system appeared in the more upfield region at δ 3.22 and 3.03 (H_a-29 and H_b-29, J = 6.4 Hz). In the HETCOR spectrum these protons were correlated to the same methylene carbon resonating at δ 51.94 (C-29). The molecular formula of **2** was determined by hreims to be $C_{30}H_{44}O_4$ (*m/z* 468.325537, calcd 468.323960). Thus, compound **2** has 30 carbons and nine degrees of unsaturation. Having no C=C bond and no additional carbonyl function, compound **2** must have an additional ring when compared with **1**. As in the case of compound **1**, a fragment ion at *m/z* 205 was observed in the eims of **2**, indicating that there was no change in rings A and B. The additional 16 mass units must be due to an additional oxygen, which is located either in ring D or ring E. Selective INEPT experiments were then carried out to determine the location of the additional ring and oxygen function. When the upfield H_b-29 (δ 3.03) was irradiated, three signals were enhanced: a carbonyl at δ 174.06 (C-30), a methine at δ 39.66 (C-19), and a quaternary carbon at δ 56.79 (C-20; $^2J_{CH}$). Additionally, irradiation of H-21 (δ 5.16) led to the enhancement of signals at δ 174.06 (C-30), 56.79 (C-20), and 48.74 (C-18). Finally, a methylene at δ 51.94 (C-29) and a methine at δ 36.58 (C-13) were enhanced when H-19 (δ 2.67) was irradiated. These data clearly showed that C-19, -20, -21, and -30 are involved in the lactone moiety, which was further found to be α-oriented by NOESY experiments as described above. The fact that both C-20 and C-29 signals were shifted from the olefinic resonating region (as in compound **1**) to the oxygen-bearing region suggested that the additional oxygen atom had formed an epoxide (additional ring) at C-20 and C-29. Further observations that C-18 and C-19 were more upfield, while C-30 was more downfield, in **2** than in **1**, could be explained by the replacement of the olefinic exomethylene by a 20,29-epoxide. The structure was thus established as 20,29-epoxy-3-oxolupan-30,21α-olide [**2**].

Ochraceolide C [**3**] had functional groups similar to those of **1**, except for an additional ketone (δ 211.04). This agreed with the hreims, which displayed a molecular ion at *m/z* 466.308443 (for $C_{30}H_{42}O_4$, calcd 466.308310), 14 mass units higher than that of **1**. The ir spectrum showed a broad band at 1710 cm^{-1} for the two ketone groups. The fragment ion at *m/z* 205, observed in the eims of **1** and **2**, was not observed in that of **3**; instead, there was an intense peak at *m/z* 219, which was also 14 mass units higher than the former. Such evidence implied that the additional ketone is present either in ring A or ring B. Comparison of the ^{13}C-nmr data with those of related compounds (14) suggested that the additional ketone was located at C-6. This fact was supported by the ^{1}H nmr, in which H-5 appeared as a singlet at δ 2.47 and H-7α as a doublet centered at

δ 2.48, meaning that no vicinal protons were attached to C-6. The ^{13}C-nmr data, in addition, indicated that both C-5 and C-7 had been significantly deshielded (by about 10 and 18 ppm, respectively), consistent with an introduction of a ketone function at C-6. The structure was thus established as 3,6-dioxolup-20(29)-en-30,21α-olide [**3**].

All three compounds were evaluated for in vitro cytotoxic potential with P-388 (murine lymphocytic leukemia), UISO-BCA-1 (breast cancer), UISO-COL-2 (colon cancer), KB-3 (human oral epidermoid carcinoma), KB-V1 (a multidrug-resistant cell line derived from KB-3), HT-1080 (human fibrosarcoma), UISO-MEL-2 (melanoma), and UISO-LUC-1 (lung cancer) cell cultures according to established protocols (17–19). As summarized in Table 2, ochraceolides A [**1**] and C [**3**] exhibited significant cytotoxic activities with cultured P-388 cells, demonstrating ED_{50} values of 0.26 and 0.53 μg/ml, respectively. They exhibited less intense activity with other cancer cell lines and were not active with KB-V1 cells (ED_{50} more than 20 μg/ml). Ochraceolide B [**2**], on the other hand, was less active, exhibiting ED_{50} values of 7.8 and 5.2 μg/ml with P-388 and KB-3 cells, respectively. No significant activity was observed with the other cell lines (ED_{50} more than 20 μg/ml). Though no conclusion can be made regarding the structure-activity relationship of the lupane lactones at this point, it appears that the presence of an α,β-unsaturated lactone may be important for the observed biological activity, since the 20,29-epoxide in **2** resulted in a reduction of in vitro cytotoxicity.

TABLE 2. In Vitro Cytotoxic Activity of Ochraceolides A [**1**], B [**2**] and C [**3**].[a]

Cell line	Compound		
	1	**2**	**3**
P-388	0.26	7.8	0.53
UISO-BCA-1	10	>20	4.5
UISO-COL-2	6.8	>20	9.2
KB-3	2.7	5.2	5.5
KB-V1	>20	>20	>20
HT-1080	9.6	>20	8.6
UISO-MEL-2	8.3	>20	8.3
UISO-LUC-1	12	>20	12

[a]The results are given as ED_{50} values (μg/ml).

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. All nmr spectra were obtained in $CDCl_3$ with TMS as an internal standard. ^{1}H-nmr and homonuclear spin decoupled spectra were obtained on a Varian XL-300 spectrometer operating at 300 MHz. Standard Varian pulse sequences were used to obtain ^{1}H-^{1}H COSY, DQCOSY, and ^{1}H-^{1}H NOESY spectra. Broad-band decoupled ^{13}C-nmr, APT, SFORD, and HETCOR spectra were recorded using either Varian XL-300 (75.44 MHz) or a Nicolet NMC-360 (90.8 MHz) spectrometer. Selective INEPT experiments were performed on the latter instrument. Uv spectra were taken on a Beckman DU-7 spectrometer and ir spectra on a Nicolet MX-1 FT-IR instrument. Optical rotation was measured on a Perkin-Elmer 241 polarimeter. Low resolution eims was recorded on Varian MAT-112S mass spectrometer at 70 eV. High resolution eims was measured with a Finnigan MAT-90 spectrometer. Cc was done on Si gel 60 (70–230 mesh, E. Merck, Darmstadt, Germany). Tlc was performed on aluminum-backed Kieselgel 60 F_{254} pre-coated plates (E. Merck). Spots were visualized by spraying with 1% vanillin in concentrated H_2SO_4, followed by heating at 110°. All solvents were redistilled before use.

PLANT MATERIAL.—The stem bark of *K. ochracea* was collected in July 1988 on Palawan Island, Philippines, and identified by one of us (D.D.S.) and Dr. D.A. Madulid (Philippine National Herbarium, Manila, Philippines). Voucher specimens (Soejarto and Madulid 6098) have been deposited at the her-

barium of the Field Museum of Natural History, Chicago, and at the Philippine National Herbarium, Manila.

EXTRACTION AND ISOLATION.—Air-dried, powdered plant material (5 kg) was extracted exhaustively by maceration/percolation at room temperature with petroleum either (4 × 7 liters). The extracts were pooled, filtered and evaporated to dryness under reduced pressure at 45° to give an orange residue (35 g). The marc was further extracted with MeOH (4 × 7 liters). After evaporation to dryness, the MeOH extract gave an orange-brown residue (320 g). The latter was then partitioned between $CHCl_3$ (3 × 2 liters) and H_2O (2 liters). Both the petroleum ether extract and the $CHCl_3$ phase were cytotoxic, whereas the aqueous layer and the interphase were not active. Having shown cytotoxicity and similar tlc patterns, the petroleum ether extract and $CHCl_3$ phase were combined (188 g) and subject to cc (Si gel). The column was eluted with mixtures of solvents of increasing polarity, starting with petroleum ether, followed by petroleum ether/EtOAc, EtOAc, EtOAc/MeOH, and MeOH. A total of 120 fractions (1 liter each) were collected and pooled, based on tlc profiles, to give eight major fractions (F1–F8). Cytotoxic activity was detected in fractions F3–F6. Fraction F3 was eluted from the column with petroleum ether-EtOAc (8:2). Following solvent removal, the residue (65 g) was redissolved in a small volume of $CHCl_3$. Ochraceolide A [**1**] was obtained by precipitation, following the addition of MeOH to the $CHCl_3$ solution of F3, and recrystallization from $CHCl_3$/MeOH (15 g, ca. 0.3% yield). Elution of the column with petroleum ether-EtOAc (6:4) gave fraction F4 (40.5 g), which on repeated cc and crystallization gave ochraceolide B [**2**] (282 mg, ca. 0.006% yield) and ochraceolide C [**3**] (190 mg, ca. 0.004% yield).

OCHRACEOLIDE A [**1**].—Colorless crystals ($CHCl_3$/MeOH); mp 223–225°; $[\alpha]^{25}D$ +31° (c = 0.1, MeOH); uv (MeOH) λ max (ε) 211 nm (6867); ir (KBr) ν max 2987–2862, 1758 (γ-lactone), 1700 (ketone), 1653, 1457, 1384, 1256, 1128, 1056, 1037, 812, 662 cm^{-1}; ^1H nmr δ 6.34 (1H, d, J = 2 Hz, H_a-29), 5.71 (1H, d, J = 1.7 Hz, H_b-29), 4.95 (1H, ddd, J = 8, 7.3, 7.1 Hz, H-21), 3.14 (1H, m, J = 10.6, 8, 2, 1.7 Hz, H-19), 2.47 (2H, m, H-2), 2.19 (1H, dd, J = 12.8, 7.3 Hz, H-22β), 1.10 (3H, s, Me-26), 1.08 (3H, s, Me-23), 1.04 (3H, s, Me-24), 0.96 (3H, s, Me-25), 0.91 (3H, s, Me-27), 0.84 (3H, s, Me-28); eims m/z (rel. int.) $[M]^+$ 452 (29), $[M-Me]^+$ 437 (5), 409 (5), 367 (9), 354 (4), 233 (11), 219 (11), 205 (37), 187 (12), 41 (100); hreims m/z $[M]^+$ 452.329444 ($C_{30}H_{44}O_3$ requires 452.329045); ^{13}C nmr see Table 1.

OCHRACEOLIDE B [**2**].—Colorless crystals (Et_2O); mp 236–238°; $[\alpha]^{25}D$ + 10° (c = 0.1, MeOH); uv (MeOH) λ max 204 nm (end absorption); ir (KBr) ν max 2972–2875, 1778 (γ-lactone), 1700 (ketone), 1457, 1386, 1266, 1250, 1162, 1118, 1025, 1000, 738, 662 cm^{-1}; ^1H nmr δ 5.16 (1H, ddd, J = 8.5, 7.7, 7 Hz, H-21), 3.22 (1H, d, J = 6.4 Hz, H_a-29), 3.03 (1H, d, J = 6.4 Hz, H_b-29), 2.67 (1H, dd, J = 10.5, 8.5 Hz, H-19), 2.44 (2H, m, H-2), 2.27 (1H, dd, J = 13, 7.7 Hz, H-22β), 1.90 (1H, m, H-1), 1.78 (1H, ddd, J = 11, 10.6, 3.5, H-18), 1.07 (3H, s, Me-23), 1.06 (3H, s, Me-26), 1.02 (3H, s, Me-24), 0.97 (3H, s, Me-27), 0.92 (3H, s, Me-25), 0.82 (3H, s, Me-28); eims m/z (rel. int.) $[M]^+$ 468 (17), $[M-Me]^+$ 453 (5), 382 (5), 370 (4), 341 (4), 219 (5), 206 (13), 205 (36), 187 (7), 41 (100); hreims m/z $[M]^+$ 468.325537 ($C_{30}H_{44}O_4$ requires 468.323960); ^{13}C nmr see Table 1.

OCHRACEOLIDE C [**3**].—Colorless crystals (toluene/petroleum ether): mp 236–238°; $[\alpha]^{25}D$ −25° (c = 0.1, MeOH); uv (MeOH) λ max (ε) 209 nm (8196); ir (KBr) ν max 2960–2850, 1760 (γ-lactone), 1710 (br, ketone), 1656, 1457, 1387, 1362, 1268, 1228, 1127, 1050, 1025, 812, 662 cm^{-1}; ^1H nmr δ 6.34 (1H, d, J = 2 Hz, H_a-29), 5.72 (1H, d, J = 1.6 Hz, H_b-29), 4.97 (1H, ddd, J = 7.2, 6.9, 7.1 Hz, H-21), 3.17 (1H, m, J = 10.8, 7.3, 1.6 Hz, H-19), 2.77 (1H, ddd, J = 14.6, 14.4, 5.9 Hz, H-2), 2.48 (1H, d, J = 11.9 Hz, H-7α), 2.47 (1H, s, H-5), 1.49 (3H, s, Me-24), 1.16 (3H, s, Me-25), 1.14 (3H, s, Me-26), 1.09 (3H, s, Me-23, 1.02 (3H, s, Me-27), 0.84 (3H, s, Me-28); eims m/z (% rel. int.) $[M]^+$ 466 (42), $[M-Me]^+$ 451 (7), 409 (12), 233 (30), 219 (26), 217 (21), 201 (12), 189 (9), 165 (29), 41 (100); hreims m/z $[M]^+$ 466.308443 ($C_{30}H_{42}O_4$ requires 466.308310); ^{13}C nmr see Table 1.

CYTOTOXICITY ASSAYS.—P-388 and HT-1080 cell lines were purchased from ATCC. Four cell lines (UISO-BCA-1, UISO-COL-2, UISO-MEL-2, and UISO-LUC-1) were established from primary human tumors in the Division of Surgical Oncology, University of Illinois College of Medicine at Chicago. KB-3 and KB-V1 were provided by Dr. Igor B. Roninson, Department of Genetics, University of Illinois College of Medicine at Chicago. KB-V1 was developed from KB-3 cells, by treatment of the latter with sublethal doses of vinblastine over an extended period of time, as reported by Shen *et al.* (20). In vitro cytotoxicity assays were performed according to the established protocols as previously described (17–19). The results were expressed as ED_{50} values (μg/ml), concentrations that inhibited cell growth by 50% after treatment for 48 h (P-388 cells) or 72 h (all other cell lines).

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